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14. ABSTRACT

Exosomes are tiny vesicles that carry information from one body cell type to another. We proposed that exosomes released from epithelial cells or macrophages in response to asbestos exposure can carry the information to mesothelial cells enabling the development of malignant mesothelioma (MM). We also hypothesized that exosomes released from primary MM tumors may contribute to growth and metastasis of primary tumors. Our work so far demonstrated that exosomes released from asbestos-exposed epithelial cells carry a different proteomic signature than exosomes from unexposed epithelial cells. Fluorescent-labelled exosomes injected into the tail vein of mice showed the presence of exosomes from asbestos-exposed epithelial cells into omentum, whereas exosomes from unexposed cells were not found in the omentum. This significant finding shows that exosomes released from asbestos-exposed epithelial cells carry a specific signature that leads them into mesothelial cells (omentum). Presently we are studying the effect of exosomes from asbestos-exposed epithelial cells in transformation of mesothelial cells, a process that may lead to MM. We also examined exosomes secreted from primary MM cells in mice, and found no significant effect of these exosomes on MM tumor growth and metastasis in this short term experiment.

15. SUBJECT TERMS
Malignant mesothelioma, exosomes, asbestos, biomarkers, mesothelial cell, epithelial cell

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1. Introduction

Exosomes are tiny vesicles (40-150 nm) that help communicate information from one body cell type to another. In the present proposal we hypothesized that exosomes play a significant role in the development of malignant mesothelioma (MM) by carrying the information from asbestos-exposed epithelial cell/macrophages to mesothelial cells. We also proposed that exosomes released from primary MM tumors influence the metastasis of these tumors. The purpose of these studies was to understand the development of MM, to characterize biomarkers of asbestos exposure, and to identify the target molecule(s) for potential treatment of MM. Successful completion may result in identification of biomarkers for early diagnosis of MM and may also help to design therapeutic strategies for this deadly disease.

2. Keywords

Malignant mesothelioma, exosomes, asbestos, biomarkers, mesothelial cell, epithelial cell

3. Accomplishments

What were the major goals of the project?

Specific Aim # 1a: To determine that exosomes secreted from asbestos-exposed human macrophages and lung epithelial cells can transform human mesothelial cells to develop MM.
We hypothesize that exosomes from asbestos-exposed lung cells carry the information to educate mesothelial cells to develop MM.
Rationale: As MM develops in the cells remotely present from initial exposure sites of asbestos fibers, exosomes, which have been shown to be carriers of information to adjacent or distant location, may be involved in MM development. There is a strong rationale to test this approach.

Specific Aim # 1b: To show the bio-distribution of fluorescently-labeled exosomes prepared from asbestos- exposed macrophages or epithelial cells in SCID mice.
We hypothesize that exosomes carrying information from asbestos exposed lung epithelial cells or macrophages diffuse into blood and/or lymph nodes and eventually reach both pleural and peritoneal mesothelial cells to develop MM.
Rationale: As exosomes have been shown to diffuse into blood and travel to distant targets, we believe that exosomes from asbestos-exposed lung cells may end up in the pleural or peritoneal cavity.

Specific Aim # 2: To show that exosomes are important for progression of primary MM tumors.
We hypothesize that exosomes secreted from mouse MM cells (#40) can enhance the progression and local metastasis of mouse MM tumors in an intraperitoneal model of MM.
Rationale: Studies performed by many groups show that exosomes secreted from tumor cells carry the information to enhance the progression and metastasis of tumor cells by various mechanisms such as angiogenesis, drug resistance, etc. (23). It has also been reported that tumor-secreted

exosomes may create a metastatic niche at the distant site (24). A report using mouse MM cells showed that exosome-pulsed dendritic cells can be used as immunotherapy for mouse MM (22). Taking all this information into account, it is logical that exosomes play important roles in tumorigenesis and can be projected as an important target for MM therapy.

Specific Aim # 3: **To profile the exosome signature from normal individuals, asbestos-exposed with benign lesions, lung cancers and MM patients' blood.**
We hypothesize that asbestos-exposed individuals will have a common signature of exosomes similar to exosomes obtained from human macrophages and epithelial cells exposed to asbestos in Aim # 1a.
Rationale: We believe that if exosomes are the carrier of the information to adjacent or distant cells, and this is the mechanism of MM development after asbestos exposure, the signature of exosomes from Aim #1a should match (to some extent) that of plasma exosomes from asbestos-exposed individuals. The findings of this aim will open the possibility of using plasma exosome signatures for early diagnosis of MM.

What was accomplished under these goals?

As part of Aim 1a, we isolated exosomes from human epithelial cells (BEAS2B) exposed to crocidolite asbestos for 72h using ultracentrifugation. These exosomes were characterized by using enhanced dark field microscopy, dynamic light scattering, and transmission electron microscopy (TEM) (Figure 1).

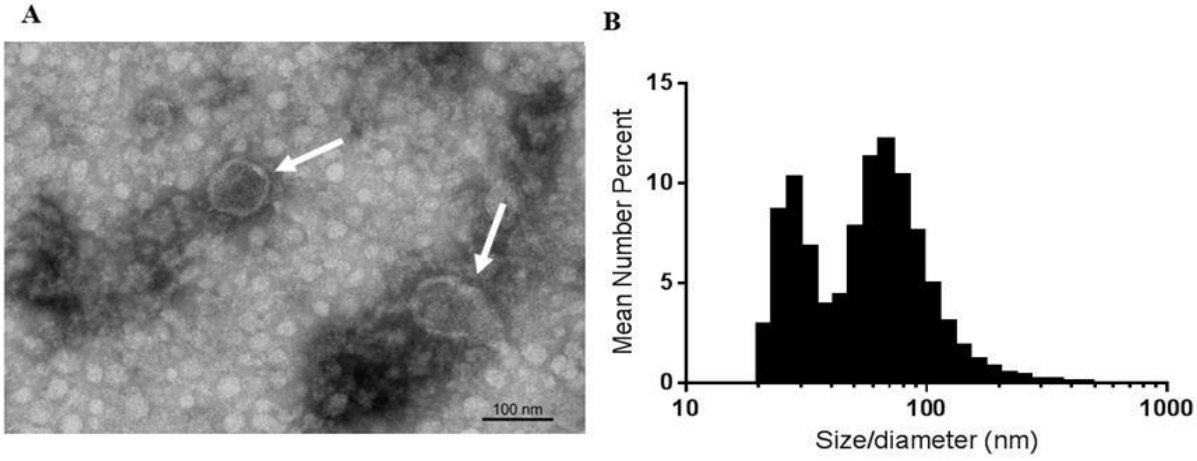

Figure 1. Characterization of exosomes from human epithelial cells (BEAS2B). **A**. Transmission electron microscopy (TEM) showing exosomes. **B**. Dynamic light scattering (DLS) showing the size of the exosomes.

Proteomic analysis performed on these exosomes at the UVM Proteomic facility suggested that asbestos exposure results in exosomes with different protein signatures (listed inTable 1).

Histone H4 OS=Homo sapiens GN=HIST1H4A PE=1 SV=2	H4_HUMAN
Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3	K1C9_HUMAN
Isoform 2 of Clathrin heavy chain 1 OS=Homo sapiens GN=CLTC	sp|Q00610-2|CLH1_HUMAN (+1)
Clathrin heavy chain 2 OS=Homo sapiens GN=CLTCL1 PE=1 SV=2	sp|P53675|CLH2_HUMAN
Desmoplakin OS=Homo sapiens GN=DSP PE=1 SV=3	sp|P15924|DESP_HUMAN
Cluster of Histone H2A type 1-B/E OS=Homo sapiens GN=HIST1H2AB PE=1 SV=2 (H2A1B_HUMAN)	H2A1B_HUMAN [4]
Tripeptidyl-peptidase 2 OS=Homo sapiens GN=TPP2 PE=2 SV=1	Q5VZU9_HUMAN
Transitional endoplasmic reticulum ATPase OS=Homo sapiens GN=VCP PE=1 SV=4	TERA_HUMAN
Cluster of Heterochromatin protein 1-binding protein 3 OS=Homo sapiens GN=HP1BP3 PE=1 SV=1 (sp|Q5SSJ5|HP1B3_HUMAN)	sp|Q5SSJ5|HP1B3_HUMAN [2]
Thrombospondin-1 OS=Homo sapiens GN=THBS1 PE=1 SV=2	TSP1_HUMAN
Integrin alpha-3 OS=Homo sapiens GN=ITGA3 PE=1 SV=5	sp|P26006|ITA3_HUMAN
DNA topoisomerase 1 OS=Homo sapiens GN=TOP1 PE=1 SV=2	TOP1_HUMAN
Integrin beta-1 OS=Homo sapiens GN=ITGB1 PE=1 SV=2	sp|P05556|ITB1_HUMAN
Filaggrin-2 OS=Homo sapiens GN=FLG2 PE=1 SV=1	FILA2_HUMAN
X-ray repair cross-complementing protein 6 OS=Homo sapiens GN=XRCC6 PE=1 SV=2	XRCC6_HUMAN
Peroxidasin homolog OS=Homo sapiens GN=PXDN PE=1 SV=2	sp|Q92626|PXDN_HUMAN
60 kDa heat shock protein, mitochondrial OS=Homo sapiens GN=HSPD1 PE=1 SV=2	CH60_HUMAN
L-lactate dehydrogenase B chain OS=Homo sapiens GN=LDHB PE=1 SV=2	LDHB_HUMAN
T-complex protein 1 subunit theta (Fragment) OS=Homo sapiens GN=CCT8 PE=3 SV=1	H7C4C8_HUMAN
Poly [ADP-ribose] polymerase 1 OS=Homo sapiens GN=PARP1 PE=1 SV=4	PARP1_HUMAN
Prostaglandin F2 receptor negative regulator OS=Homo sapiens GN=PTGFRN PE=1 SV=2	FPRP_HUMAN
Isoform 3 of Immunoglobulin superfamily member 8 OS=Homo sapiens GN=IGSF8	sp|Q969P0-3|IGSF8_HUMAN (+1)
40S ribosomal protein S3 OS=Homo sapiens GN=RPS3 PE=2 SV=1	E9PPU1_HUMAN
Prolactin-inducible protein OS=Homo sapiens GN=PIP PE=1 SV=1	PIP_HUMAN
Cystatin-A OS=Homo sapiens GN=CSTA PE=1 SV=1	CYTA_HUMAN

Table 1. Proteomic profiling performed on exosomes isolated from asbestos-exposed and control human epithelial cells (BEAS2B) demonstrate increased amount of proteins in response to asbestos exposure. Text in red shows proteins involved in the process of cancer.

These exosomes were then labelled with green fluorescent dye and injected into SCID mice via the tail vein. Lung, pleura, and omentum were collected from these mice and analyzed for the presence of exosomes. Tissue analysis showed that exosomes from asbestos-exposed epithelial cells home into omentum, whereas exosomes from non-asbestos-exposed epithelial cells were not detected in omentum (Figure 2). This interesting finding suggests that exosomes secreted from asbestos-exposed epithelial cells possess a signature that targets them to omentum (home for mesothelial cells). Other tissues are being processed to analyze the presence of exosomes.

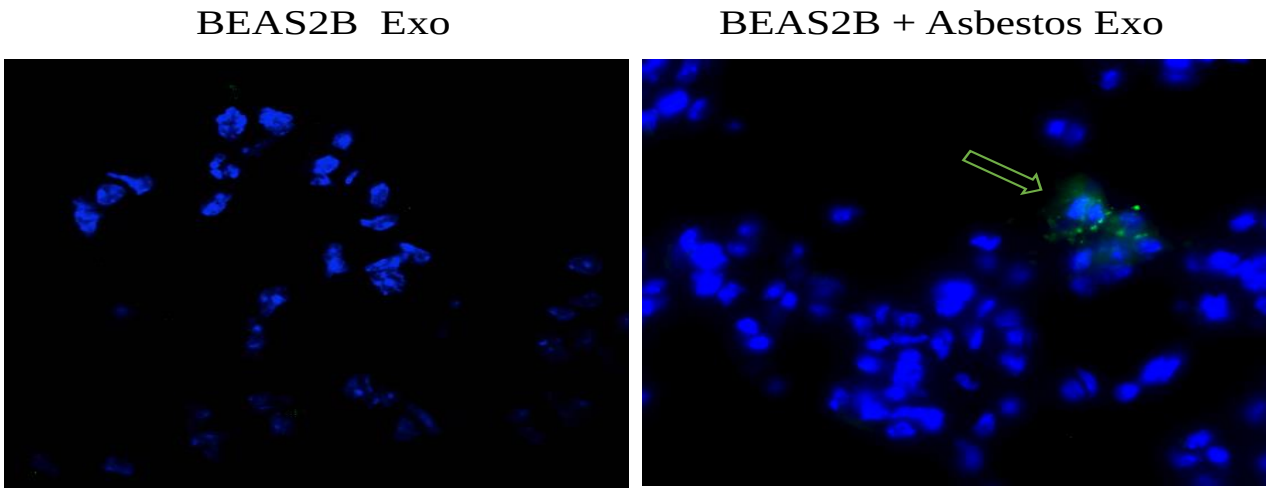

Figure 2. Exosomes from asbestos-exposed epithelial cells accumulate into omentum of mice. Arrow pointing to green fluorescent exosomes. Blue- omentum tissue.

Thus far in Aim 2, we isolated exosomes from mouse mesothelioma cell # 40 and performed proteomic analysis under two different growth conditions (0.5% FBS or 10% FBS). We observed that the protein profile of exosomes changes drastically when grown under stressed conditions (0.5% FBS) compared to normal conditions (10% FBS). We then injected exosomes collected from mouse MM cells #40 grown in 10% exosome free FBS into C57/BL6 mice bearing peritoneal MM tumors at an injection frequency of 5 times/week for 3 weeks. Tumor growth and inflammation in the peritoneal cavity were measured. We did not observe an effect of exosomes on tumor growth or inflammation in this preliminary experiment. We are currently designing more thorough, longer term experiments with and without chemotherapeutic drugs to assess this further.

Based on the data generated we can draw several conclusions. First, exosomes generated from epithelial cells exposed to asbestos contain a special proteomic signature that may be responsible for their uptake by mesothelial cells, thus causing mesothelioma. Second, exosomes released from stressed cancer cells have a more distinct proteomic signature than unstressed cancer cells. Finally, in a short-term experiment exosomes released from primary MM tumors had no significant effect on MM tumor growth.

What opportunities for training and professional development has the project provided?

Phillip Munson is a graduate student currently enrolled in the UVM Cellular, Molecular, and Biomedical Sciences program. He joined my lab in June 2015 and is now in training on this project. He has developed skills to isolate exosomes from different types of cells and characterize them by different methods. He is also receiving training to develop his grant and manuscript writing skills.

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

For Aim 1a we will prepare exosomes from epithelial cells and macrophages with and without exposure to asbestos and add them to human mesothelial cells for 48h. We will assess mesothelial to fibroblastic transition (MFT) using standard protocol in our lab. Results of this experiment will help us understand if exosomes derived from asbestos exposed epithelial cells or macrophages can transform distantly present mesothelial cells and help in development of mesothelioma.

For Aim 1b, we will finish analysis of other collected tissues for labeled exosomes.

For Aim 3, we are waiting subcontract site to get IRB and HPRO approval so as to get started with human plasma samples for exosome isolation and proteomic profiling.

4. Impact

What was the impact on: Development of the principal discipline(s) of the project? Other disciplines? Technology transfer? Society beyond science and technology?

Nothing to report

5. Changes/Problems

This award involves a subcontract with Weill Cornell Medical College, with Hector Peinado Selgas, Ph.D. as Co-PI. Subsequent to the award start date, Dr. Selgas accepted a position at the Spanish National Cancer Center (CNIO) in Madrid, Spain and has moved there. Because of this circumstance, we proposed to DoD that the majority of the work outlined in the SOW for the subcontract will now be conducted at Cornell under new leadership with Dr. David Lyden, a senior investigator, initially named as a consultant for the project. We plan to initiate and execute a second subcontract with CNIO Spain for Dr. Selgas (5% effort and some research money) whereby he will supervise research and communicate regularly with Dr. Lyden to ensure the appropriate progression of the project. All relevant paperwork for this new subcontract was submitted to DoD for approval on April 15, 2015 and we are currently awaiting a response in order to initiate the subcontract. Because the subcontract has not yet begun, collaborators do not have adequate data to share at this point.

Changes in approach and reasons for change
Work in aim 3 has not started due to delay in subcontract approval.

Actual or anticipated problems or delays and actions or plans to resolve them
The only problem is delay in subcontract approval that is delaying this project. All required documents were submitted to DoD on April 15, 2015 and we are waiting for approval to start the project work at subcontract sites.

Changes that had a significant impact on expenditures

50% of the total project money is allocated to subcontract, which cannot be used unless work at subcontract site starts. Also, money allocated for training of personnel from site 1 (UVM) at site 2 (Cornell) could not be used due to the same reason.

Significant changes in use or care of human subjects
Because of delay in start of subcontract we don't have update about IRB approval for Aim3 (site 2 is responsible for collecting normal human plasma for the project). Once IRB is approved HPRO will be submitted to DoD for final approval).

Significant changes in use or care of vertebrate animals / use of biohazards and/or select agents
No changes

6. Products

Publications, conference papers, and presentations

Journal Publication: Munson PB, Shukla A. Recent advances in targeted therapy in medicine. *Medicines;* 29 Sept 2015 [In process] Invited review; federal support acknowledged.

Books or other non-periodical, one-time publications / Other publications, conference papers, and presentations:
Nothing to report

Website(s) or other internet site(s) / Technologies or techniques / Inventions, patent applications, licenses / Other products:
Nothing to report

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

1. Arti Shukla, Ph.D. (Principal Investigator) – No change
2. Maximilian Macpherson – No change
3. Brooke Mossman – No change
4. Phillip Munson (graduate student) – new person since last report who worked for 6 months isolating and characterizing exosomes, and performing related experiments. Mr. Munson's efforts were funded by the UVM Cellular, Molecular, and Biomedical Sciences program.

Has there been a change in the active other support of the PD/PI or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Weill Cornell Medical School
Department of Pediatrics Division of Hematology/Oncology

515 East 71st Street
New York, NY

Partner's contribution to the project: Collaboration

8. Special Reporting Requirements

Collaborative awards

Major changes to the subcontract are awaiting DoD approval (described in Item 5 above). The request for this approval was submitted by UVM to DoD on April 15, 2015 and we have followed up on several occasions. To date, we have not received any response from DoD; therefore, work has not yet been initiated with our contracted collaborators.

9. Appendices

None